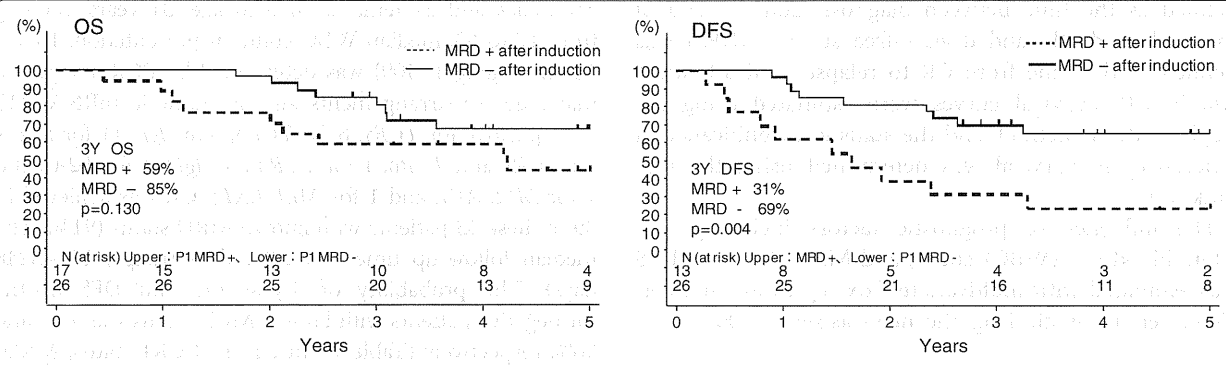

Figure 1 Impact of post-induction minimal residual disease (MRD) status on overall survival (OS) and disease-free survival (DFS). Patients who were MRD negative after induction therapy (first course A) (n = 26) had a significantly better 3-year DFS compared with those who were MRD positive (n = 13) (69% vs. 31%, p = 0.004).

Since relapse in ALL patients leads to very poor prognosis [24-26], the notion that allogeneic HSCT should be performed for all patients with ALL beyond CR1 is difficult to be realized in clinical situations [7].

The international ALL trial MRC UKALL XII/ECOG E2993 showed that allogeneic HSCT using matched related donors provided survival benefit for standard-risk adult patients with Ph-negative ALL in CR1 compared with chemotherapy, while there was no significant survival benefit for high-risk patients. Allogeneic HSCT is able to reduce relapse rates in both standard-risk and high-risk patients; however, there is a decrease in OS in the high-risk patients because of their higher rates of transplant-related mortality. The high-risk in this international study was defined as having as 1 of the following factors: age more than 35 years, a high WBC count at presentation (>30 × 10^9/L for B lineage and >100 × 10^9/L for T lineage) [23]. Age is a significant prognostic factor for ALL patients receiving allogeneic HSCT as well as chemotherapy [27]. Therefore, allogeneic HSCT may not be a recommended option for patients defined as high-risk because of their age being more than 35 years old [28,29].

Recent studies have shown that a pediatric-inspired ALL chemotherapy protocol significantly improves treatment outcome in relatively young adult ALL patients [30-34], and this patient population is at standard-risk in terms of age. Thus, the indication of allogeneic HSCT based on the risk stratification made by initial presentation needs to be tested, and more reliable indication for allogeneic HSCT in adult patients with Ph-negative ALL in CR1 is necessary.

MRD measurement in adult patients with Ph-negative ALL has been reported to be useful for identifying patients with a significantly high risk of relapse. The German Multicenter Study Group for adult ALL (GMALL) study used PCR analysis of antigen-receptor genes to assess MRD in standard-risk ALL patients. Low-risk patients

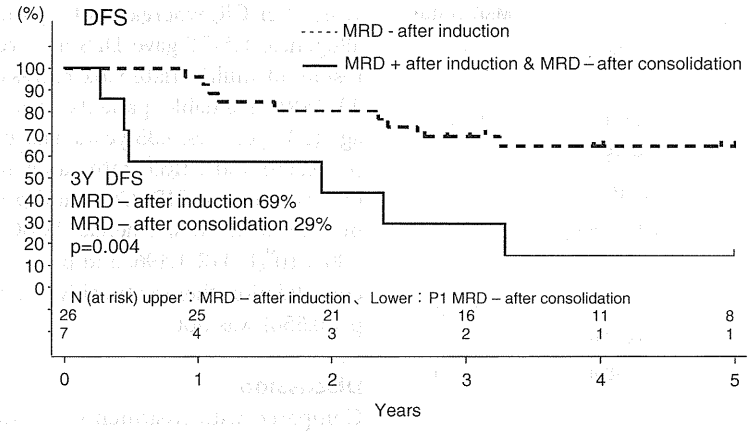

Figure 2 Effect of time taken to become minimal residual disease (MRD)-negative on disease-free survival (DFS). Patients who were MRD positive after induction and became MRD negative after consolidation chemotherapy C in the first course (n = 7) had a significantly worse DFS compared with patients who were MRD negative after induction chemotherapy A in the first course (n = 26) (69% vs. 29%, p = 0.004).

Table 2 Multivariate analysis for disease-free survival (Cox Regression Model)

	Hazard ratio	95% CI		P-value
Risk factors				
Age	5.067	1.616	15.885	0.005
WBC	1.496	0.457	4.897	0.505
MRD status after induction	8.769	2.465	31.196	<0.001
MRD status after consolidation	0.67	0.18	2.492	0.55

Age: ≥35 vs <35.
WBC: $\geq 30\times10^9$/L vs $<30\times10^9$/L.
MRD after Induction: positive vs. negative.
MRD after consolidation: positive vs. negative

were those with MRD–negative on days 11 and 24 and had a 3-year relapse rate of 0%; high-risk patients were those with MRD-positive until week 16 and had a relapse rate of 94% [10]. The Northern Italy Leukemia Group-ALL 09/00 study found that MRD was the most significant predictor of relapse [13]. The estimated 5-year DFS was 72% in 58 MRD-negative patients at the end of consolidation and 14% in 54 MRD-positive patients.

Our results indicate that patients with MRD negativity after induction therapy provided excellent DFS without allogeneic HSCT, whereas patients with MRD positivity after several consolidation therapies showed very poor DFS if they did not undergo allogeneic HSCT. This observation is in line with above reports. Our analysis showed that late-attained MRD negativity could not lead to good prognosis, while other groups reported the MRD negativity at the end of consolidation to be associated with good prognosis. This controversy may reflect the sensitivity level of MRD measurement. We used semi-quantitative PCR analysis and defined MRD negativity as $<1 \times 10^{-3}$, whereas GMALL [10] and The Northern Italy Leukemia Group-ALL 09/00 study [13] analyzed MRD according to EuroMRD-ALL guidelines [35,36] and considered $<1 \times 10^{-4}$ as MRD negativity. In our study, MRD positivity after the second course of consolidation was seen in 6 of 37, while MRD positivity at the end of consolidation was observed in 54 of 112 in The Northern Italy Leukemia Group-ALL study 09/00 [13]. Thus, our MRD-negative patients had a possibility of MRD positivity if more sensitive MRD analysis was used. We suggest these late-attained MRD-negative patients were potential candidates for allogeneic HSCT.

In this study, the results of multivariate Cox regression analysis for DFS indicates that age (≥35 years vs. <35 years) and MRD status after induction therapy were significant prognostic factors, whereas WBC count ($\geq 30 \times 10^9$/L vs. $<30 \times 10^9$/L) or MRD status after consolidation therapy was not. Age is one of the most important prognostic factors in adult ALL patients, and age of our study population was median 31 years-old ranging 17 to 63 including adolescent and young adult patients. Thus, these relative young patients were supposed to have good prognosis with chemotherapy. Initial WBC count has been another important prognostic factor in adult ALL patients, but not in our study. Our chemotherapy regimen was modified CALGB 19802 with dose intensification of daunorubicin and cytarabine, and it might be possible that this intensive chemotherapy conquer negative impact of high initial WBC count. According to the recently reported result of CALGB 19802 [16], age (≥60 years vs. <60 years) was a significant prognostic factor for DFS, while initial WBC count ($\geq 30 \times 10^9$/L vs. $<30 \times 10^9$/L) was not. This report was in line with our observation. In our analysis MRD status after induction therapy (positive vs. negative: HR 8.769, and $p < 0.001$) was a very strong prognostic factor for DFS. Whether negative impact of MRD positivity could be overcome by allogeneic HSCT is the next consideration. There are two reports regarding the effect of prospective allocation for allogeneic HSCT based on MRD positivity in adult patients with Ph-negative ALL in CR1.

In the Northern Italy Leukemia Group-ALL study 09/00, for the MRD-positive patients at the end of consolidation, there was a significantly better 4-year DFS for 36 patients who had an allogeneic (n = 22) and autologous (n = 14) HSCT compared to 18 patients unable to undergo HSCT (33% vs. 0%, p = 0.0000) [13]. The GMALL reported that 5-year DFS for MRD-positive patients at week 16 with (n = 57) vs. without (n = 63) allogeneic HSCT were 44 ± 8% vs. 11 ± 4% respectively (p <0.0001) [37]. In our study, among MRD-positive patients following consolidation chemotherapy C in the first course, all of 3 patients without allogeneic HSCT relapsed while 1 of 3 patients with allogeneic HSCT did. The size of our study population was too small for statistical analysis. However, these three studies clearly indicate that MRD-positive patients at late phase of chemotherapy have little chance of DFS more than 10% without allogeneic HSCT [37]. These MRD-defined high-risk patients had much worse prognosis compared with conventional high-risk patients defined by initial presentation. Furthermore, allocation of allogeneic HSCT could improve the prognosis of MRD-defined high-risk patients.

The interpretation of our results may be affected by a limited number of adult ALL patients. A role of MRD measurement should be evaluated in relation with patients' geography, chemotherapy regimens used, and timing and sensitivity of MRD analysis. With our less sensitive MRD analysis compared to EuroMRD-ALL guidelines, we could identify patients with good early treatment response not indicated for allogeneic HSCT, while we could not identify patients with good late treatment response. In near future, the assessment of MRD status using standardized protocols and RQ-PCR [35,36] will be a valuable tool to stratify a risk of relapse in adult patients with Ph-negative ALL in CR1.

In conclusion, our data suggest that evaluation of MRD at least twice after induction and consolidation is very useful when considering clinical indication for allogeneic HSCT in adult patients with Ph-negative ALL in CR1.

Competing interests
The authors declare no competing financial interests.

Authors' contributions
All authors recruited and treated patients for this study. DNA-baed MRD analysis in this report were performed under supervision of SY. KN and MH were involved in the drafting of the manuscript. MH coordinated the study. All authors reviewed and approved the final draft of the manuscript.

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Quantitation of hematogones at the time of engraftment is a useful prognostic indicator in allogeneic hematopoietic stem cell transplantation

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TRANSPLANTATION

Quantitation of hematogones at the time of engraftment is a useful prognostic indicator in allogeneic hematopoietic stem cell transplantation

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Key Points

- Quantitation of hematogones at engraftment is useful to predict prognosis of patients treated with allogeneic stem cell transplantation.

Transient marrow expansion of normal B-cell precursors, termed hematogones, is occasionally observed after hematopoietic stem cell transplantation (HSCT). To understand the clinical significance of this phenomenon, we enumerated hematogones in 108 consecutive patients who received allogeneic HSCT for the treatment of hematologic malignancies, including acute myelogenous leukemia, advanced myelodysplastic syndromes, acute lymphoblastic leukemia, and non-Hodgkin lymphoma. Hematogone quantitation was performed at the time of complete donor engraftment (median day 25 and 32 in patients who received bone marrow and cord blood cell transplants, respectively). Hematogones were polyclonal B cells, and their frequencies correlated positively with blood B-cell numbers, and inversely with donors' but not recipients' age, suggesting that hematogones reflect cell-intrinsic B-cell potential of donor cells. Interestingly, patients developing hematogones that comprised > 5% of bone marrow mononuclear cells constituted a group with significantly prolonged overall survival and relapse-free survival, irrespective of their primary disease or donor cell source. In addition, patients with > 5% hematogones developed severe acute graft-versus-host diseases less frequently, which may contribute toward their improved survival. We therefore conclude that the amount of hematogones at the time of engraftment may be a useful tool in predicting the prognosis of patients treated with allogeneic HSCT. (*Blood.* 2013;121(5):840-848)

Introduction

Hematogones are transient increases in lymphoblast-looking cells in the bone marrow.[1,2] Because of the morphologic resemblance between residual leukemic clones and hematogones, expansion of hematogones during the recovery phase from chemotherapy and bone marrow transplantation occasionally causes diagnostic confusion.[1-3] Phenotypic analyses have demonstrated that hematogones are normal B-cell precursors, including pro-B, pre-B, and immature B cells coexpressing CD10 and CD19.[1,2] The fact that hematogones become prominent in the recovery phase after chemotherapy or hematopoietic stem cell transplantation (HSCT)[1-6] suggests that they could reflect active B-cell reconstitution. They are also sometimes seen in steady-state hematopoiesis, especially in healthy infants and young people.[2,4,7,8] Previous work demonstrated that in the recovery phase after chemotherapy, the percentage of hematogones in the bone marrow was inversely correlated with patients' age.[1] However, it is unclear whether the age-associated decline in hematogones frequency reflects cell-intrinsic defects of hematopoietic stem cell activity or cell-extrinsic defects such as aging of the bone marrow microenvironment.

Recent reports have shown that hematogone expansion correlates with favorable outcomes in acute myelogenous leukemia (AML) patients treated with chemotherapy[5] or cord blood transplantation (CBT).[6] However, the precise number or frequency above which hematogones correlate with clinical significance has not been clarified. Previous reports[1,5,6] have reported hematogone frequency relative to bone marrow mononuclear cells (MNCs), total nuclear cells (TNCs), and frequencies of B-cell precursors, and as a result, hematogone expansion has been described with frequencies ranging from > 0% to 0.9%.[1,5,6]

To better understand the etiology and clinical significance of hematogones, we measured percentages of B-cell precursors in the bone marrow via flow cytometry in 108 consecutive patients with hematologic malignancies, including AML, advanced myelodysplastic syndromes (MDS), acute lymphoblastic leukemia (ALL), and lymphoma, who achieved successful engraftment after allogeneic HSCT at our institution. The analysis of hematogones was performed on the day of engraftment, defined as the day when circulating granulocytes reached > 0.5×10^9/L for 3 consecutive days,[9-12] and the bone marrow showed complete donor-type chimerism via polymerase chain reaction (PCR) analysis. To minimize the effect of expanding granulocytes on hematogone frequencies, bone marrow MNCs were used for flow cytometric

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Table 1. Patients' characteristics

	BMT: no. (%), [range]	CBT: no. (%), [range]	Total (%)	*P*
Recipient sex, male/female	35 (32)/24 (22)	26 (24)/23 (21)		NS
Recipient age, y	49.2 (mean) [20-66]	47.3 (mean) [19-67]		NS
Donor sex, male/female	37 (34)/22 (20)	24 (22)/25 (23)		NS
Donor age, y	36.7 (mean) [17-66]	0		< .001
No. of infused cell, /kg	2.80×10^8 (mean) [0.92-4.02]	0.28×10^8 (mean) [0.18-0.50]		< .001
Primary disease				NS
AML/advanced MDS				
CR	14 (13)	11 (10)	25 (23)	
non-CR	21 (19)	14 (13)	35 (32)	NS
Total	35 (32)	25 (23)	60 (56)	
ALL				
CR	3 (3)	3 (3)	6 (6)	
non-CR	2 (2)	10 (9)	12 (11)	NS
Total	5 (5)	13 (12)	18 (17)	
Lymphoma				
CR	7 (6)	3 (3)	10 (9)	
non-CR	12 (11)	8 (7)	20 (19)	NS
Total	19 (18)	11 (10)	30 (28)	
Conditioning regimen				< .01
TBI/CY	28 (26)	24 (22)		
BU/CY	14 (13)	0 (0)		
RIC	17 (16)	25 (23)		
GVHD prophylaxis				< .001
TAC + sMTX	51 (47)	6 (6)		
CSP + sMTX	8 (7)	28 (26)		
CSP + MMF	0 (0)	15 (14)		
HLA disparity				< .001
6/6	39 (36)	1 (1)		
5/6	20 (19)	7 (6)		
4/6	0 (0)	22 (20)		
3/6	0 (0)	19 (18)		
Days required for engraftment	25 (median) [15-32]	32 (median) [14-39]		< .01

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BMT, bone marrow transplantation; BU, busulfan; CBT, cord blood transplantation; CR, complete remission; CSP, cyclosporine; CY, cyclophosphamide; GVHD, graft- versus-host disease; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; NS, not significant; RIC, reduced-intensity conditioning; sMTX, short-term methotrexate; TAC, tacrolimus; and TBI, total body irradiation.

analyses in all cases. Our data suggest that the number of hematogones generally reflects cell-intrinsic B-cell potential of donor hematopoietic stem cells (HSCs) and that this declines with aging. We also found that hematogone frequencies of > 5% of total MNCs is a useful cutoff line to distinguish patient groups with significantly better overall survival (OS) or with relapse-free survival (RFS), irrespective of their primary diseases or donor cell sources. We propose that the quantitation of hematogones at engraftment may be useful to predict the prognosis of patients treated with allogeneic HSCT.

Methods

Patients

From 2005 to 2010, 134 patients with high-grade hematologic malignancies were treated with allogeneic HSCT in Kyushu University Hospital. These patients included AML cases with high risk,[13] relapsed or refractory status, advanced MDS cases with intermediate-II or high risk on International Prognostic Scoring System classification,[14,15] ALL cases with high risk,[16] relapsed or refractory status, and relapsed non-Hodgkin lymphoma cases. Within these 134 patients, grafts were rejected in 5 cases and residual malignant cells proliferated soon after HSCT in 21 cases, without achieving successful engraftment. The remaining consecutive 108 cases, in which allogeneic HSCT was successful and complete donor-type chimerism was documented, were enrolled in this study. Fifty-nine and 49 patients received bone marrow transplantation (BMT) and CBT, respectively. Patients' characteristics are summarized in Table 1. This study was approved by the institutional review board of Kyushu University Hospital and conducted in accordance with the Declaration of Helsinki.

Evaluation of remission status before HSCT

Before HSCT, patients were intensively searched for residual malignant cells to define their pretransplantation remission status. In acute leukemia or advanced MDS cases, bone marrow samples were checked first by microscopic analysis, and were subjected to multicolor flow cytometric analysis.[13,17] Complete remission (CR) was diagnosed when percentages of cells of leukemia phenotype were < 0.5% in the bone marrow. Furthermore, 21 patients with acute leukemia or MDS had leukemia-specific genes such as BCR-ABL, FLT3-ITD, AML1-ETO, and MLL fusions, and PCR amplification of these genes were used to detect minimal residual disease (MRD).[13] Within these 21 patients, 17 patients were diagnosed as CR based on flow cytometric analyses. CR results for these 17 patients were also confirmed by PCR. In lymphoma patients, remission status was defined by evaluating the involvement of lymphoid organs using FDG-PET CT scan and/or MRI methods, and was also defined by evaluating the involvement of bone marrow by flow cytometry, as previously described.[18]

Transplantation procedures

Patients' characteristics were not statistically different between BMT and CBT recipient groups in terms of sex, age, and primary disease (Table 1). Conditioning regimen consisted of total body irradiation/cyclophosphamide (CY) for 28 BMT and 24 CBT recipients, busulfan (BU)/CY for 14 BMT recipients, and fludarabine-based reduced-intensity conditioning[19,20] for 17 BMT and 25 CBT recipients, respectively (Table 1).

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Prophylaxis for graft-versus-host disease (GVHD) was tacrolimus/short-term methotrexate (sMTX) for 51 BMT and 6 CBT recipients, cyclosporine (CSP)/ sMTX for 8 BMT and 28 CBT recipients, and CSP/mycophenolate mofetil for 15 CBT recipients (Table 1). The mean number of donor cells transplanted was 2.8×10^8/kg in BMT recipients and 0.28×10^8/kg in CBT recipients. Bone marrow units were obtained from the Japan Marrow Donor Program or related donors, and cord blood units were obtained from the Japanese Cord Blood Bank Network.

Evaluation for engraftment

The bone marrow sampling for the analysis of hematogones was performed when patients achieved successful engraftment. The standard criterion for engraftment was used according to previous studies.[9-12] Blood neutrophil numbers were checked daily after transplantation, and the successful engraftment was defined when neutrophils exceeded 0.5×10^9/L for 3 consecutive days. When patients met the criteria for engraftment, host/donor microchimerism analysis was performed (see the next section). If the analysis showed complete donor type chimerism, hematogones in the bone marrow were counted by multicolor flow cytometric analysis.

Chimerism analysis

To analyze donor/recipient cell chimerism, PCR amplification of polymorphic short tandem repeats (STR) was performed to confirm engraftment of donor cells. PCR using synthesized oligonucleotide templates were performed using TAKARA *Taq* Reagent Kits and run in the Perkin Elmer GeneAmp PCR system 9600 or 2400. The donor-cell origin and recipient-cell origin PCR product mixture was loaded onto the 373A sequencer (Applied Biosystems) with a size marker, and the data were processed using the GeneScan software (Applied Biosystems) as described previously.[21]

Flow cytometry analysis and cell sorting

The bone marrow mononuclear cells were prepared by the gradient centrifugation method as previously described.[22,23] Cells were stained with allophycocyanin-conjugated anti-CD34 (BD Pharmingen), biotin-conjugated anti-CD38 (Caltag Laboratories), FITC-conjugated anti-CD10 (DAKO), PE-conjugated anti-CD20 (BD Biosciences), PE-Cy7–conjugated anti-CD19 (BioLegend), and Cy5-PE–conjugated lineage (Lin) mixture (anti-CD3, -CD4, -CD8 (BD Pharmingen) -CD11b (Caltag Laboratories), -CD14, and -CD56 (Beckman Coulter).[22-25] Streptavidin-conjugated Cy7-allophycocyanin (BD Pharmingen) was used for visualization of biotinylated antibodies. For analysis of mature B cells, peripheral blood (PB) cells were stained with FITC-conjugated anti-CD10 (DAKO), PE-conjugated anti-CD20 (BD Biosciences), PE-Cy7–conjugated anti-CD19 (BioLegend), and Cy5-PE–conjugated Lin mixture. Available PB cells at day 90 after HSCT could be obtained from 64 patients and evaluated. Cells were analyzed by using a FACSAria (BD Biosciences) or FACSCanto (BD Biosciences). Cell sorting was performed on a 5-color FACSAria (BD Biosciences). To minimize contamination, cells were collected after the second round of sorting using sorting gates identical to those used in the first-round sorting. Definition of hematogones is a series of normal B-lymphoid precursors, including $CD34^+CD38^+CD10^+CD19^+Lin^-$ pro-B cells, $CD34^{-/lo}CD38^+CD10^+CD19^+$ pre-B cells, and $CD34^-CD38^+CD10^+CD19^+$ $CD20^+$ immature B cells[26-28] in bone marrow MNCs. Isotype controls were used to define the cutoff of positivity of each antigen on a FACS.

PCR analysis of *IGH* gene rearrangement

To analyze clonality of *IGH* gene rearrangements status of hematogones, DNA was obtained from 10 000 double-sorted cells[22,23,29] from all recipients presenting $> 0.1\%$ MNCs of hematogones on FACS. Then PCR amplification of DJ_H and VDJ_H gene rearrangement was performed as described previously.[24,30]

Statistical analysis

Relationships of percentages of hematogones with age, the day of engraftment, and numbers of circulating B lymphocytes were analyzed with the Spearman rank correlation analysis. Comparison between 2 groups or condition was tested with the Mann-Whitney U test. The categorical variables were analyzed with the 2-tailed χ^2 test. Survival was plotted with Kaplan-Meier curves, taking the interval from date of HSCT to death/relapse or last contact. Comparisons between each group were performed with the log-rank test and the Cox proportional hazards model. Univariate analysis was performed with logistic or exact logistic regression, and the parameters that present $P < .20$ were reevaluated by multivariate analysis.[31] Multivariate analysis was performed with logistic regression applying Firth's bias reduction. A P value $< .05$ was considered to be statistically significant.

Results

Hematogones that appeared at the time of engraftment are polyclonal B-cell precursors

One hundred eight consecutive cases treated with successful allogeneic BMT or CBT were enrolled in this study. Hematogones in the bone marrow were counted on the day of engraftment by multicolor flow cytometric analysis. The successful engraftment was judged when neutrophils exceeded $> 0.5 \times 10^9$/L for 3 consecutive days.[9-12] At this phase, it is critical to exclude residual leukemic cells or host-derived B-cell precursors from a cell fraction of hematogones. To this end, polymorphic STR was amplified to test the host/donor microchimerism, and only when patients' bone marrow consisted of 100% donor-derived cells, the analysis of hematogones was performed. The complete donor-type chimerism verifies that host-derived normal hematopoietic cells and malignant leukemic cells have been eliminated below the sensitivity of FACS,[32-34] and therefore that phenotypically defined hematogones in these patients on FACS were donor-derived normal cells.

Hematogones were morphologically blastic cells (Figure 1A), and were identified by surface phenotype, according to the definition of pro-B, pre-B, and/or immature B cells that coexpress CD10 and CD19 on their cell surface[1,26-28] (Figure 1B). To minimize the effect of granulocytes on hematogone frequencies, we used MNCs instead of TNCs in our analysis. The frequencies of hematogones in MNCs are usually higher than those in TNCs (not shown), as reported previously.[1]

The time median to engraftment was 25 and 32 days in patients treated with BMT and CBT, respectively (Table 1). The time required for engraftment appears to be consistent with previous reports.[34-39] Percentages of B-cell precursors within the bone marrow MNCs at the time of complete donor-type engraftment were significantly higher in CBT recipients than in BMT recipients (6.37% vs 1.75%; $P < .001$; Figure 1C). There was no significant relationship between the day of engraftment (the day of sampling) and the frequency of hematogones (Figure 1D).

In 106 of 108 patients who had $> 0.1\%$ of B-cell precursors in the bone marrow MNCs, B-cell precursors were purified by a multicolor FACS and were subjected to *IGH* rearrangement analysis. In all of these patients, B-cell precursors were polyclonal based on the rearrangement analysis of the *IGH* genes (Figure 1E).

Hematogones generally represent B-cell recovery potential of the graft and their emergence is related to age of donors but not recipients

Because hematogones are normal B-cell precursors, we tested whether the presence of a high number of them could reflect the active B-cell recovery after HSCT. FACS analysis of circulating blood cells revealed that the frequency of bone marrow B-cell precursors was significantly correlated with the number of blood

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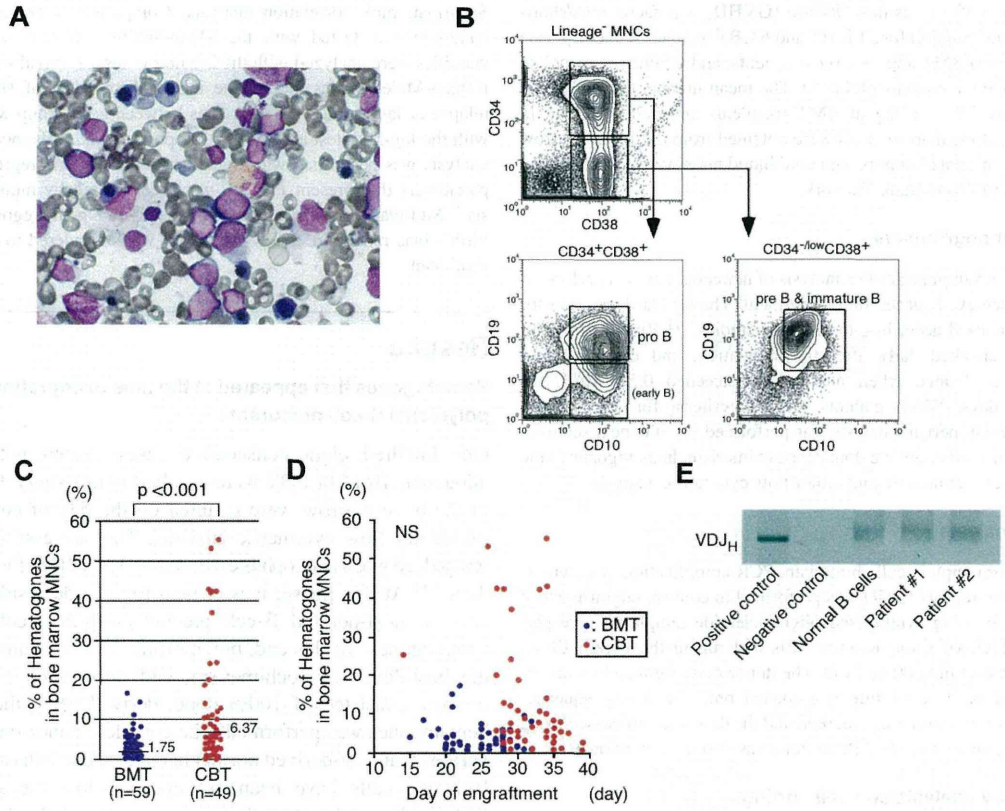

Figure 1. Detection of hematogones after allogeneic HSCT. (A) Typical appearance of hematogones in the bone marrow after HSCT (Giemsa staining ×1000; OLYMPUS BH-2 microscope [Olympus]; ACT-2U imaging software [Nikon]; 27°C). (B) Evaluation of hematogones on a 5-color FACS. Hematogones are defined as MNCs coexpressing CD10 and CD19 in the bone marrow at engraftment. They include $CD34^+CD38^+CD10^+CD19^+Lin^-$ pro-B cells, $CD34^{-/lo}CD38^+CD10^+CD19^+$ pre-B cells, and $CD34^-CD38^+CD10^+CD19^+CD20^+$ immature B cells. (C) Percentage of hematogones in the bone marrow MNCs in patients who received BMT and CBT. CBT recipients presented much higher frequency of hematogones compared with BMT recipients ($P < .001$). Solid bars indicate the median percentage of hematogones for each recipient; MNC, mononuclear cells; BMT, bone marrow transplantation; and CBT, cord blood transplantation. (D) The relationship between the day of engraftment and percentages of hematogones. There was no relationship between these parameters. (E) *IGH rearrangement analysis of purified hematogones. B-cell precursors were polyclonal in all* 106 recipients analyzed.

B cells at the time of engraftment ($R = 0.47$, $P < .001$; Figure 2A), and with those even on day 90 after HSCT ($R = 0.22$, $P < .01$; Figure 2B). These results suggest that expansion of hematogones reflects not only enhanced B-cell reconstitution potential of the graft, but also prolonged B cell–producing capability of donor HSCs.

The age of BMT donors ranged from 17 to 66 years old (median, 37 years; Table 1). Interestingly, there was a significant inversed correlation between donor age and percentage of bone marrow hematogones in patients treated with BMT ($R = 0.32$, $P = .02$; Figure 2C blue line). When the age of CBT donors were defined as 0-year old, the significant inversed correlation between age and hematogone numbers was also found in all patients entered in this study ($R = 0.42$, $P < .001$; Figure 2C black line). In contrast, recipients' age and hematogone numbers did not show any relationship (Figure 2D). Furthermore, as shown in Table 2, the time of engraftment was not affected by primary diseases of patients, or by their remission status at the time of HSCT. Thus, although the patients who fail to achieve CR are usually treated with higher total doses of chemotherapeutic drugs because of their refractory disorders, it did not affect the day of engraftment or the day of hematogone analysis for this study. These data strongly suggest that the number of hematogones after HSCT generally reflects the cell-intrinsic B-cell recovery potential of donor HSCs, which may decline by aging.

The emergence of hematogones up to > 5% of MNCs in the bone marrow represents a good prognosis for patients treated with allogeneic HSCT

It should be critical to draw a line of hematogone numbers to distinguish a group of patients with clinical significance. Therefore, we first compared the OS and RFS among patient subgroups with ≤ 1%, 1%-2%, 2%-3%, 3%-4%, 4%-5%, or > 5% of hematogones in our study (Figure 3A-B). Strikingly, patients who developed hematogones up to > 5% of MNCs showed significantly better 3-year OS (100%) and RFS (93.3%), compared with any other group. Patient groups with ≤ 1%, 1%-2%, 2%-3%, and 3%-4% of hematogones showed similar 3-year OS and RFS that were 37%-53% and 22%-51%, respectively. Interestingly, patients with 4%-5% hematogones appeared to show intermediate levels of OS (86%) and RFS (64%), although this is not statistically better than those in patients with ≤ 1% hematogones (Figure 3A-B). Based on these results, we hypothesized that the development of > 5% of hematogones might be critical to distinguish a patient group with favorable prognosis.

According to this criteria, 43 patients developed > 5% MNCs of hematogones (HG^+) and the remaining 65 patients had ≤ 5% MNCs of hematogones (HG^-). As shown in Figure 3A, in HG^+ patients, 3-year OS was 100%, whereas in HG^- patients, it was 45% ($P < .001$). The favorable OS in HG^+ groups is at least

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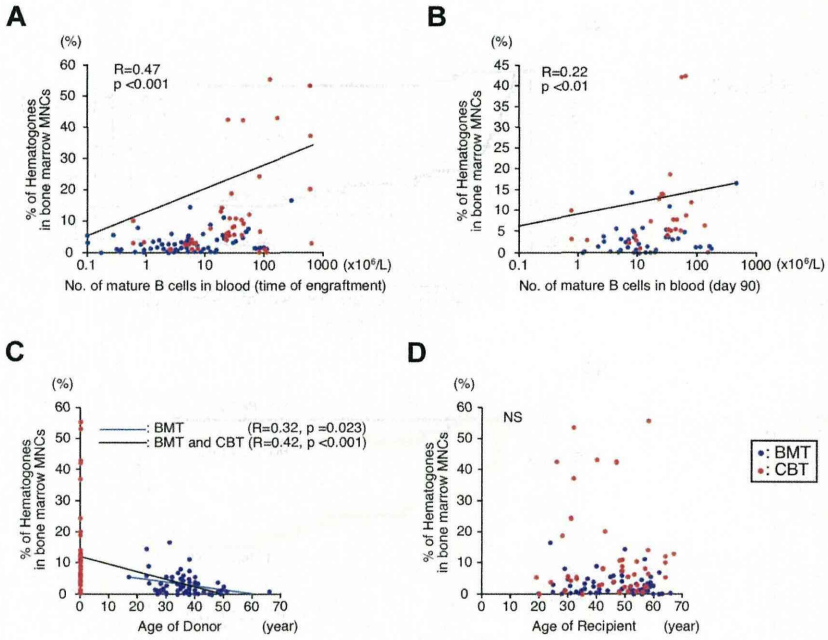

Figure 2. Analysis of hematogones, and the correlation of their frequency compared with blood B-cell numbers and age of donors. (A) A relationship between frequencies of hematogones and blood B cells at engraftment ($P < .001$). (B) A relationship between frequencies of hematogones at engraftment and blood B cells on day 90 ($P < .01$). (C) A relationship between frequency of hematogones and donor's age in patients who received BMT (blue line, $P = .023$), and in all recipients treated with either BMT or CBT (black line, $P < .001$). (D) No significant relationship was observed between frequency of hematogones and recipient age. NS indicates not significant.

because of the less frequent disease relapse. As shown in Figure 3B, significant association was observed between the presence of hematogones and 3-year RFS after HSCT: 3-year RFS was 93% and 37% in HG^+ and HG^- patients, respectively ($P < .001$). The association between the presence of $> 5\%$ hematogones and favorable OS and RFS was also seen when the analysis was performed in patient subgroups that received either BMT or CBT (Figure 3C-D). These data strongly suggest that the emergence of hematogones is a useful predictor of favorable outcomes at least in terms of OS and RFS, irrespective of donor cell source.

The emergence of hematogones (> 5% of MNCs) marks favorable outcomes for allogeneic HSCT especially in patients who failed to achieve complete remission, irrespective of primary malignant disease

We then analyzed whether the good prognosis designated by the emergence of hematogones is dependent on the primary malignant disorder. The OS and RFS were analyzed in each patient group with AML/advanced MDS, ALL, or non-Hodgkin lymphoma. As shown in Figure 4, HG^+ patients always showed significantly better OS and RFS compared with HG^- patients, in any of these patients groups suffering from different primary diseases.

It is well known that the achievement of CR at the time of transplantation favorably affects the prognosis after allogeneic HSCT.[13] Interestingly, in AML/advanced MDS patients, the HG^+ group showed significantly prolonged OS and RFS compared with the HG^- group, irrespective of their remission status at HSCT (Figure 5). The similar analysis was performed in ALL and lymphoma patient groups (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Although each group contained only a limited number of patients, statistically significant prolonged OS and RFS were also seen in patients who did not achieve CR at HSCT in both the ALL and the lymphoma patient groups.

Thus, the appearance of hematogones might mark favorable OS and RFS regardless of their primary malignancy.

Expansion of hematogones is frequently observed in patients who did not develop infection or severe acute GVHD

In this study, all 43 HG^+ patients are currently alive, although primary diseases have relapsed in 3 patients. In contrast, 32 of 65 HG^- patients have died. The causes of death in these 32 HG^- patients are shown in Table 3. Twenty-six patients died of their refractory primary disease, and 6 patients died of TRM, including

Table 2. Time required for engraftment in patients grouped by their primary disease or complication of infection or acute GVHD

		Time required for engraftment, d					
		BMT			CBT		
Disease	Remission status	No.	Mean, d	*P*	No.	Mean, d	*P*
AML and advanced MDS	CR/non-CR	14/21	27.0/24.4	.11	11/14	31.5/31.8	.37
ALL	CR/non-CR	3/2	23.7/29.5	.35	3/10	34.3/32.1	.45
Lymphoma	CR/non-CR	7/12	26.1/22.8	.10	3/8	33.0/29.3	.44
			Overall	.10		Overall	.53
After HSCT							
Infections	Yes/No	38/21	25.3/24.8	.66	22/27	30.8/32.2	.61
Acute GVHD	Grade II-IV/Grade 0-I	26/33	24.7/25.3	.25	14/35	32.3/31.3	.47

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BMT, bone marrow transplantation; CBT, cord blood transplantation; CR, complete remission; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; and MDS, myelodysplastic syndrome.

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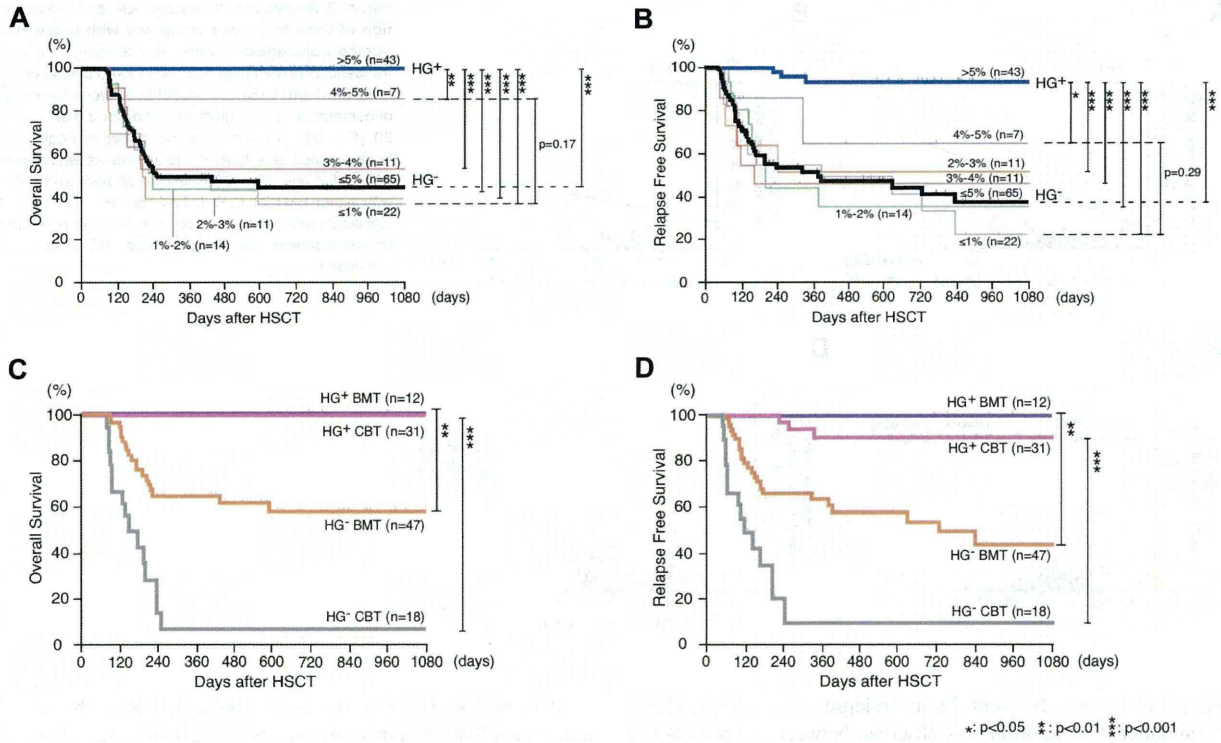

Figure 3. Patients whose hematogones comprised > 5% bone marrow MNCs constitute a group with significantly improved survival, irrespective of HSC sources. (A-B) The Kaplan-Meier estimates of (A) OS and (B) RFS among patient subgroups with ≤ 1% (gray line), 1%-2% (green line), 2%-3% (orange line), 3%-4% (red line), 4%-5% (purple line), ≤ 5% (black bold line), or > 5% (blue bold line) hematogones in the bone marrow MNCs. Forty-three patients who developed > 5% MNC hematogones (HG^+) showed significantly better 3-year OS and RFS, compared with any of each group ($P < .01$ and $P < .05$, respectively), as well as to 65 patients with ≤ 5% MNCs hematogones (HG^-; $P < .001$ for both). (C-D) The Kaplan-Meier estimates of (C) OS and (D) RFS in HG^+ and HG^- groups that received transplants with BMT or CBT. The improved OS and RFS were seen in HG^+ groups regardless of the source of HSC. HG^+ indicates patients developed hematogones (> 5% of bone marrow MNCs); HG^- indicates patients who failed to develop hematogones (≤ 5% of bone marrow MNCs).

acute GVHD (2 patients) and viral infections (4 patients). Of the 26 patients who died of primary disease, 24 developed both acute GVHD and infections.

We analyzed the relationship between the emergence of documented hematogones in the bone marrow, and variables including sex of donor/recipient, days required for engraftment, primary diseases, times of intensive chemotherapy before HSCT, remission status, conditioning regimen, documented infectious disease, and episode of acute/chronic GVHD by using univariate and multivariate analysis. These analyses were performed in patient groups treated with BMT and CBT, respectively.

There were no correlations found between the emergence of > 5% of hematogones and clinical factors such as the day of engraftment, primary disease, times of intensive chemotherapies, and remission status, in either univariate or multivariate analyses. As shown in Table 4, in univariate analysis, a hematogone increase up to > 5% of MNCs was found more frequently in patients without viral infection (such as cytomegalovirus, human herpesvirus 6, and adenovirus; BMT: $P = .03$; CBT: $P < .01$), and those did not develop severe acute GVHD of grade II-IV (BMT: $P < .01$; CBT: $P < .01$). Time required for engraftment did not differ between patient groups with or without infections, or acute GVHD (Table 2). These data appear to be compatible with the analysis of causes of death in HG^- patients (Table 3). On the other hand, in multivariate analysis, severe acute GVHD of grade II-IV, but not infection was the significant risk factor for emergence of hematogones (BMT: $P = .03$; CBT: $P = .04$; Table 4). Based on these analyses, the emergence of hematogones heralds less frequent development of severe acute GVHD.

Discussion

Hematogones are immature B-cell precursors that reside mainly in the bone marrow of every normal individual,[1,2,27,40] and their numbers could reflect activity of normal B lymphopoiesis. Hematogones are occasionally seen in large numbers in healthy people, especially in infants and young children.[2,4,7,8] Interestingly, recent reports have suggested that the presence of detectable numbers of hematogones at the recovery phase from myelosuppression reflects better prognosis of patients with AML treated with chemotherapy[5] or CBT,[6] although the underlying mechanism of this phenomenon is unclear. The increase of hematogones may reflect eradication of leukemic cells that could inhibit normal hematopoiesis,[1,5] or rapid immune reconstitution that could suppress infection and severe acute GVHD in an allogeneic HSCT setting.[6]

In these reports, the presence of hematogones was documented when they were detectable at a low frequency: ≥ 0.01% of TNCs at a recovery phase[5] or > 0% and > 0.9% of MNCs on day 21 and 100, respectively.[6] In contrast, the patient cohort in our study received allogeneic HSCT, and the majority (106 of 108 cases) of patients had > 0.1% of hematogones at engraftment by our multicolor flow cytometric analysis (Figure 1C). Therefore, it was critical to set an appropriate threshold value and timing of sampling

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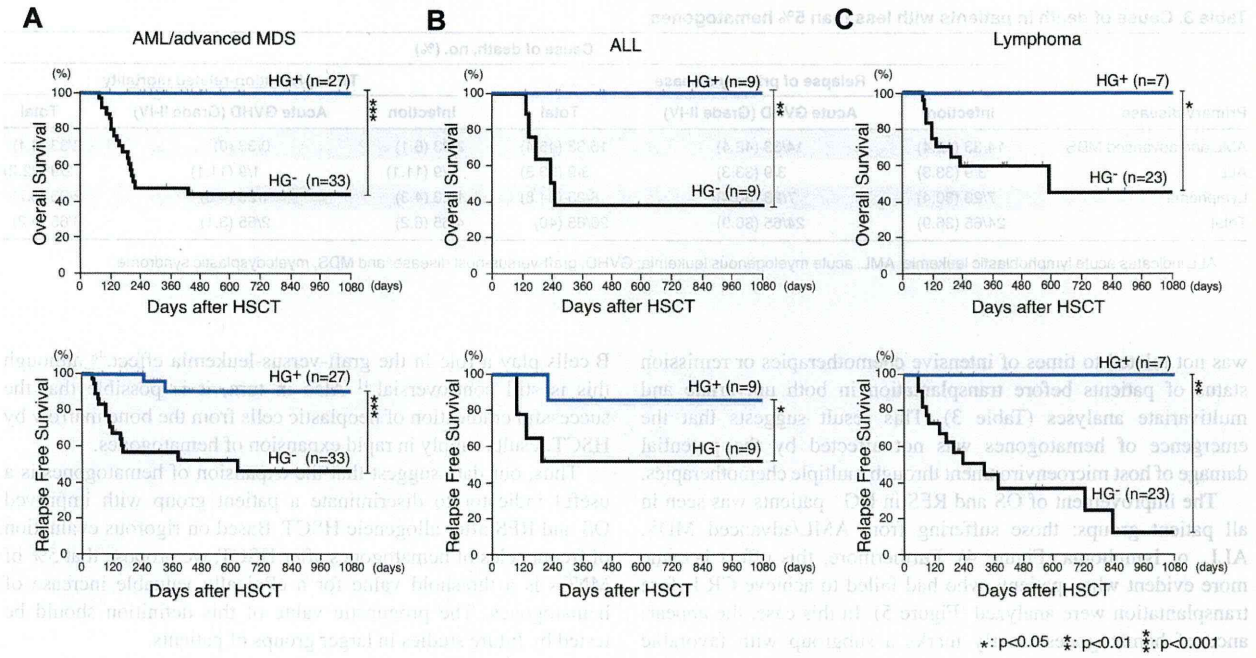

Figure 4. Patients who developed > 5% hematogones as a fraction of their MNCs constitute a group with significantly improved survival, irrespective of their primary disease. The Kaplan-Meier estimates of OS and RFS in HG^+ and HG^- patients differentiated with their primary disease. In each group of patients with (A) AML or advanced MDS, (B) ALL, and (C) lymphoma, HG^+ groups showed significantly better OS and RFS, compared with the HG^- group ($P < .001$ for both).

to decide a clinically meaningful increase of hematogones in an allogeneic HSCT setting. Furthermore, previous studies were performed only in patients with AML,[5,6] but not in patients with lymphoid neoplasms, presumably because it was difficult to discriminate a small number of neoplastic lymphoid cells from hematogones.[6]

To accurately enumerate hematogones in patients with various clinical backgrounds and with different donor cell sources, we performed the analysis on the day when patients met the clinical criteria of engraftment[9-12] and displayed complete donor-type chimerism. The confirmation for donor-type chimerism allowed us to avoid miscounting neoplastic lymphoid cells as hematogones. Because these samples should be free from host-derived cells, we included patients with lymphoid malignancies in our study. We rigorously measured the frequencies of hematogones within bone marrow MNCs by 6-color flow cytometric analysis.

In our study, donor-derived hematogones were polyclonal, based on *IGH* rearrangement analysis in all cases, and therefore the presence of hematogones should be a snapshot of normal B lymphopoiesis at the recovery phase. In fact, the frequencies of hematogones at engraftment were correlated with circulating B-cell numbers at least until day 90 (Figure 2). Importantly, we here show that the frequencies of hematogones were correlated with donors' age, but not with recipients' age, suggesting the age-dependent decline of B-cell potential of donor HSCs. This is compatible with previous mouse studies in which younger HSCs are capable of producing more abundant B cells.[41-43]

According to our criteria, the engraftment was seen on days 25 and 32 (median) in BMT and CBT groups (Figure 1D), respectively, consistent with previous studies.[35-39,44] Within each BMT or CBT group, the engraftment day was not significantly altered by the patients' primary disease or remission status at transplantation (Table 2). The bone marrow sampling for hematogone analysis was performed on the day of engraftment. As shown in Table 3, the timing of sampling (= the day of engraftment) was not significantly related to emergence of hematogones in univariate and multivariate analyses. Interestingly, however, our data suggest that when hematogones reach > 5% of MNCs at engraftment, it has a profound clinical impact on patients' OS and RFS (Figure 3A-B). It is of note that the emergence of documented hematogones

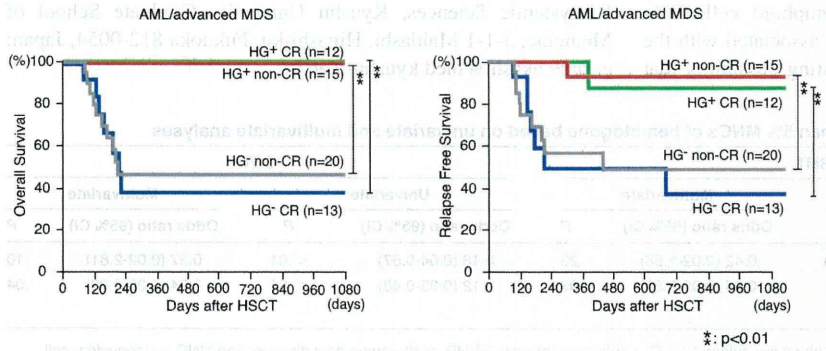

Figure 5. The presence of hematogones marks a group with good prognosis in AML/advanced MDS patients. The Kaplan-Meier estimates of OS and RFS in HG^+ and HG^- patients in AML or advanced MDS differentiated with their remission status before HSCT. Significantly better OS and RFS were seen in HG^+ groups irrespective of their remission status.

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Table 3. Cause of death in patients with less than 5% hematogones

	Cause of death, no. (%)					
	Relapse of primary disease			Transplantation-related mortality		
Primary disease	Infection	Acute GVHD (Grade II-IV)	Total	Infection	Acute GVHD (Grade II-IV)	Total
AML and advanced MDS	14/33 (42.4)	14/33 (42.4)	15/33 (45.4)	2/33 (6.1)	0/33 (0)	2/33 (6.1)
ALL	3/9 (33.3)	3/9 (33.3)	3/9 (33.3)	1/9 (11.1)	1/9 (11.1)	2/9 (22.2)
Lymphoma	7/23 (30.4)	7/23 (30.4)	8/23 (34.8)	1/23 (4.3)	1/23 (4.3)	2/23 (8.7)
Total	24/65 (36.9)	24/65 (36.9)	26/65 (40)	4/65 (6.2)	2/65 (3.1)	6/65 (9.2)

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; GVHD, graft-versus-host disease; and MDS, myelodysplastic syndrome.

was not related to times of intensive chemotherapies or remission status of patients before transplantation in both univariate and multivariate analyses (Table 3). This result suggests that the emergence of hematogones was not affected by the potential damage of host microenvironment through multiple chemotherapies.

The improvement of OS and RFS in HG^+ patients was seen in all patient groups: those suffering from AML/advanced MDS, ALL, or lymphoma (Figure 4). Furthermore, this effect became more evident when patients who had failed to achieve CR before transplantation were analyzed (Figure 5). In this case, the appearance of hematogones clearly marks a subgroup with favorable OS and RFS, irrespective of their primary diseases. In contrast, in patients who had achieved CR before transplantation, prolonged OS and RFS were found only in patients with AML/advanced MDS, but not in patients with ALL or lymphoma (Figure 5). A larger study including higher number of patients should be performed to clarify the impact of hematogones on HSCT results in CR patients with lymphoid malignancies.

The analyses of risk factors for the appearance of > 5% MNCs of hematogones revealed that in both BMT and CBT patients, the less frequent occurrences of severe acute GVHD and infections were significantly correlated in univariate analyses, whereas the less frequent severe acute GVHD was the only risk factor in multivariate analyses (Table 3). As shown in Table 4, all 32 deaths occurred only in HG^- patients, and 24 of these 32 patients developed both severe acute GVHD and infection before the relapse of the disease. In these patients, doses of immunosuppressive drugs were escalated to control acute GVHD, which might cause development of infections as well as recurrence of primary disease.[6,45,46] It is therefore possible that less frequent development of severe acute GVHD in HG^+ patients is one of the reasons for their better OS and RFS.

The rapid reconstitution of the immune system represented by a high number of hematogones should be able to prevent infection.[6,45] In turn, successful prevention of acute GVHD could result in proliferation of hematogones because acute GVHD itself may suppress hematopoietic recovery by targeting the bone marrow HSC niche[47] or by attacking directly B-lymphoid cells.[46] In addition, the fact that improvement of RFS is associated with the expansion of hematogones suggests an interesting possibility that B cells play a role in the graft-versus-leukemia effect,[48] although this is still controversial.[41] Also in turn, it is possible that the successful eradication of neoplastic cells from the bone marrow by HSCT results simply in rapid expansion of hematogones.

Thus, our data suggest that the expansion of hematogones is a useful indicator to discriminate a patient group with improved OS and RFS after allogeneic HSCT. Based on rigorous evaluation of frequencies of hematogones after HSCT, we propose that 5% of MNCs is a threshold value for a clinically valuable increase of hematogones. The prognostic value of this definition should be tested by future studies in larger groups of patients.

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Authorship

Contribution: T.S. and T.M. coordinated the project, designed and performed the transplantation and experiments, analyzed the data, and wrote the manuscript; Y.K., Y.M., K. Kamezaki, K. Takenaka, H.I., K.N., T.T, and K. Kato performed the transplantation and provided technical advice; K. Takase, H.H., A.N., Y.I., T.K., and T.E. provided patient information, clinical samples, and technical advice; and K.A. designed the experiments, reviewed the data, and edited the manuscript.

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Table 4. Risk factors for development of more than 5% MNCs of hematogone based on univariate and multivariate analyses

	BMT				CBT			
	Univariate		Multivariate		Univariate		Multivariate	
	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*
Infections, Yes/No	0.19 (0.05-0.74)	.03	0.42 (0.02-1.58)	.23	0.16 (0.04-0.57)	< .01	0.37 (0.02-2.81)	.10
Acute GVHD, Grade II-IV/0-I	0.09 (0.01-0.73)	< .01	0.04 (0.00-0.85)	.03	0.12 (0.03-0.48)	< .01	0.04 (0.00-0.80)	.04

BMT indicates bone marrow transplantation; CBT, cord blood transplantation; CI, confidence interval; GVHD, graft-versus-host disease; and MNC, mononuclear cell.

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Identification of a novel HLA-A*24:02-restricted adenovirus serotype 11-specific CD8+ T-cell epitope for adoptive immunotherapy

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ABSTRACT

Subgroup B adenovirus serotype 11 (Ad11) occasionally causes fatal infections in immunocompromised patients. The present study describes a novel Ad11 epitope presented by HLA-A*24:02 that could be used for adoptive immunotherapy. Ten synthetic Ad11 hexon protein-derived nonamer peptides that bound to HLA-A*24:02 were selected by a computer algorithm and MHC stabilization assay. Stimulation of peripheral blood mononuclear cells from HLA-A*24:02+ donors with each of these synthetic peptides induced peptide-specific CD8+ T-cells for three peptides. Testing the reactivity of these peptide-specific CD8+ T-cells against various target cells confirmed that peptide TYFNLGNKF is naturally processed in Ad11-infected cells and is presented by HLA-A*24:02. Emergence of TYFNLGNKF-specific CD8+ T-cells coincided with the clearance of adenoviruses in a patient with Ad11 disease. Importantly, TYFNLGNKF-specific CD8+ T-cells were suggested to be not serotype cross-reactive. The novel HLA-A*24:02-restricted Ad11 epitope could be used for anti-Ad11 adoptive immunotherapy and to monitor immunity to Ad11 using MHC tetramers.

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1. Introduction

Adenoviruses (AdV) cause lethal infections in immunocompromised hosts such as hematopoietic stem cell transplantation (HSCT) and chemotherapy recipients (Chakrabarti et al., 2002; Leen et al., 2006a; Yokose et al., 2009). Although antiviral agents, such as ribavirin and cidofovir, have been used for the treatment of AdV infection, their efficacy is limited by weak intrinsic activity against viruses and by toxicity (Ison, 2006; Lindemans et al., 2010). Furthermore, reconstitution of AdV-specific T-cells is required for the control of AdV infection (Feuchtinger et al., 2005; Heemskerk et al., 2005). These observations have led to the development of adoptive T-cell therapy for the management of AdV infection (Feuchtinger et al., 2006; Leen et al., 2006b).

There are several different approaches to generate virus-specific T-cells for adoptive therapy. In previous reports, peripheral blood mononuclear cells (PBMCs) were stimulated with the lysate of AdV-infected cells or with adenoviral vector-transduced cells to generate AdV-specific T-cells (Feuchtinger et al., 2004; Leen et al., 2004a). However, the clinical use of these strategies is complicated by the concerns associated with transferring live viral particles to patients who are immunocompromised. Another method to generate virus-specific T-cells is to stimulate PBMCs with immunogenic peptides derived from viral proteins. This method is advantageous in that synthetic peptides can be readily produced under good manufacturing practice conditions. Furthermore, the feasibility of this approach has been documented in clinical studies in which cytomegalovirus (CMV)-pp65 peptide-specific cytotoxic

Abbreviations: AdV, adenoviruses; Ad11, adenovirus serotype 11; BIMAS, Bioinformatics and Molecular Analysis Section; CM, culture medium; CMV, cytomegalovirus; CTLs, cytotoxic T-cells; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; E:T, effector to target; FBS, fetal bovine serum; HC, hemorrhagic cystitis; HSCT, hematopoietic stem cell transplantation; IFN-γ, interferon-γ; IL-2, interleukin-2; K562/A*24:02, K562 cells transduced with HLA-A*24:02; LCLs, Epstein-Barr virus-transformed B-lymphoblastoid cell lines; LYA, LYANSAHAL; mAb, monoclonal antibody; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; TCRs, T cell receptors; TYF, TYFNLGNKF; VYS, VYSGSIPYL.

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T-cells (CTLs) were given therapeutically or prophylactically to HSCT recipients (Meij et al., 2012; Micklethwaite et al., 2007).

The wide clinical application of AdV-specific T-cells generated by stimulating PBMCs with immunogenic peptides requires knowledge of T-cell epitopes restricted by prevalent MHC molecules. Previous studies that have identified T-cell epitopes of AdV have focused exclusively on the subgroup C AdV (Leen et al., 2004b, 2008; Tang et al., 2006; Zandvliet et al., 2010). Subgroup B AdV serotype 11 (Ad11) is a major pathogen for hemorrhagic cystitis (HC) (Akiyama et al., 2001; Miyamura et al., 1989; Mori et al., 2012) and occasionally causes disseminated infection with fatal outcomes in immunocompromised patients (Taniguchi et al., 2012). However, T-cell epitopes of Ad11 have not been identified. Therefore, the goal of the following study was to identify a novel T-cell epitope of Ad11 presented by HLA-A*24:02, which is one of the most common HLA class I molecule in many ethnic groups (60% in Japanese population, 20% in Caucasians, and 12% in Africans) (Gomi et al., 1999).

2. Materials and methods

2.1. Donor and patient specimens

PBMCs and serum from HLA-A*24:02+ healthy volunteer donors and a patient with Ad11-associated HC were obtained after informed consent. In addition, urine was collected from a patient with Ad11-associated HC. Measurement of AdV DNA in the patients' serum and urine was performed by real-time polymerase chain reaction, as previously described (Funahashi et al., 2010).

2.2. Cell lines

Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (LCLs) were generated by infection of PBMCs from healthy donors with concentrated EBV-containing supernatants of cultured B95-8 cells (Leen et al., 2004b). T2-A24 cells, the transporter associated with antigen processing-deficient B and T hybrid cell line T2 transfected with the HLA-A*24:02 gene, were kindly supplied by Dr. Y. Akatsuka (Aichi Cancer Center Research Institute, Nagoya, Japan). K562 cells were transduced with retroviruses that encode CD80 and CD86 and were selected to >90% purity by cell sorting for expression of these co-stimulatory ligands. CD80 and CD86$^+$ K562 were then transduced with retroviruses that encode a full-length HLA-A*24:02 (Phoenix-Ampho System; Orbigen) and sorted twice to obtain cells of >95% purity that expressed HLA-A*24:02 (named K562/A*24:02). Cell lines were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS). For preparation of peptide-pulsed LCLs, K562, and K562/A*24:02, the cells were washed once, resuspended in RPMI-1640 medium, and pulsed with the corresponding synthetic peptide at 5 μg/ml at room temperature for 2 h. The cells were then washed once and used in stimulation assays.

2.3. Infection of K562 and K562/A*24:02 with AdV

Ad11 isolated from patients was used in the experiments. K562/A*24:02 were infected with Ad11 at a multiplicity of infection of 100 and used for enzyme-linked immunosorbent assay (ELISA) 72 h after infection. Infection was confirmed by documentation of AdV hexon antigen expression by flow cytometry.

2.4. Peptides

A computer-based program (Bioinformatics and Molecular Analysis Section (BIMAS), HLA peptide binding predictions; http://www-bimas.cit.nih.gov/molbio/hla_bind/) was used to identify potential HLA-A24-binding peptides within the Ad11 hexon protein. The nonamer peptides with a score exceeding 100 were selected and synthesized. HLA-A24-binding peptide, QYDPVAALF, derived from the human CMV-pp65 protein (Kuzushima et al., 2001), and TYFSLNNKF, derived from the human AdV serotype 5 hexon protein (Leen et al., 2004b), were also synthesized. All peptides were synthesized by Medical & Biological Laboratories (Nagoya, Japan).

2.5. MHC stabilization assay

All candidate peptides were tested for their capacity to bind to HLA-A24 molecules on the surface of T2-A24 cells as described previously (Kuzushima et al., 2001). Briefly, T2-A24 cells (3×10^5 cells) were incubated with 200 μL RPMI-1640 medium containing 0.1% FBS, 5×10^{-5} M β-mercaptoethanol (Sigma), 3 μg/ml human β2-microglobulin (Sigma), and each of the peptides at a concentration of 10 μM at 37 °C for 16 h. Following the incubation, surface HLA-A24 molecules were stained with the anti HLA-A23/A24 monoclonal antibody (mAb) and anti-mouse FITC-labeled antibodies. Expression was measured by flow cytometry, and mean fluorescence intensity (MFI) was recorded. Percent MFI increase was calculated as follows: percent MFI increase = (MFI with the given peptide − MFI without peptide)/(MFI without peptide) × 100.

2.6. Generation and expansion of peptide-specific CD8$^+$ T-cells

PBMCs obtained from healthy volunteers were placed at a concentration of 2×10^6 cells per tube in a 14 ml polypropylene tube with 1 ml of RPMI-1640 medium with 10% human serum [referred to as culture medium (CM)] and directly stimulated with peptides at a concentration of 1 μg/ml. At day 3, CM was added to a final volume of 2 ml and supplemented with 25 IU/ml recombinant human interleukin-2 (IL-2) (R&D Systems, Minneapolis, MN). Cells were transferred to a 24-well plate at day 7, re-stimulated with peptides every 7 days, cultured until day 21 or 28, and tested by interferon-γ (IFN-γ) secretion assay for the presence of peptide-specific CD8$^+$ T-cells. To expand peptide-specific CD8$^+$ T-cells, CD8$^+$ cells producing IFN-γ in the presence of peptides were isolated using IFN-γ secretion assay, followed by expansion in the presence of OKT3 mAb (Janssen Pharmaceutical), IL-2, and feeder cells, as described previously (Sugimoto et al., 2009).

2.7. Antibodies and flow cytometric analysis

All antibodies were purchased from BD Biosciences (San Jose, CA) unless otherwise noted. The following anti-human antibodies for staining of cell surface markers and intracellular molecules were used: IFN-γ-FITC, CD3-PE-Cy5.5 (Invitrogen, Carlsbad, CA), CD8-PE, -APC, or -PerCP-Cy5.5, and HLA-class I-PE (eBioscience, San Diego, CA). PE-conjugated HLA class I tetramers folded with AdV peptides were used to stain virus-specific T cell receptors (TCRs) (Medical & Biological Laboratories). In addition, mouse anti HLA-A23/A24 (One Lambda, Canoga Park, CA) and mouse anti-AdV hexon protein (Abcam, Cambridge, UK) antibodies were used in combination with anti-mouse IgG/IgM-FITC.

The IFN-γ secretion assay was performed using the IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, cells were re-stimulated for 4 h at 37 °C in the presence or absence of peptides (1 μg/ml). Cells were incubated with IFN-γ catch reagent and cultured for 45 min at 37 °C to allow for IFN-γ secretion, followed by staining with anti-IFN-γ, CD3, and CD8 antibodies. Intracellular cytokine staining assay for IFN-γ was performed as previously described with some modifications (Terakura et al., 2012). In brief, cells were re-stimulated with peptide-pulsed

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or peptide-unpulsed autologous LCLs and incubated at 37 °C for 4 h. Brefeldin A (Golgiplug, BD Biosciences) was added during the last 2.5 h of incubation to block secretion of cytokines. Subsequently, the cells were fixed, permeabilized, and stained with anti-IFN-γ and CD8 antibodies, using FIX/PERM and PERM/Wash solution (BD Biosciences). Data acquisition was performed with FACSAria or FACSCanto flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (TreeStar, Inc., Ashland, OR).

2.8. ELISA

K562/A*24:02, those loaded with peptides, and those infected with Ad11 were dispensed at 3×10^4 cells/well into triplicate cultures in 96-well, round-bottom plates. Then, 3×10^4 of peptide-specific CD8$^+$ T-cells were added to each well, and after a 24 h of co-culture at 37 °C, IFN-γ in the supernatant was measured using an ELISA method (Endogen).

2.9. Cytotoxicity assay

K562 and K562/A*24:02 loaded with or without peptides were used as target cells in cytotoxicity assay. Target cells were labeled for 2 h with ^{51}Cr, washed twice, dispensed at 1×10^3 cells/well into triplicate cultures in 96-well, round-bottom plates, and incubated for 4 h at 37 °C with peptide-specific CD8$^+$ T-cells at various effector to target (E:T) ratios. Percent-specific lysis was calculated as follows: percent-specific lysis = (experimental cpm − spontaneous cpm) × 100/(maximum cpm − spontaneous cpm).

3. Results

3.1. Selection of potential HLA-A24-binding peptides within Ad11 hexon protein

To identify potential HLA-A24-binding peptides within amino acid sequences of the Ad11 hexon protein, the amino acid sequence of the protein was analyzed by a web-based algorithm designed to predict HLA-binding peptides, based on estimation of the half-time dissociation of the HLA-peptide complex (http://www-bimas.cit.nih.gov/molbio/hla_bind/). Ten peptides with estimated half-time dissociation scores above 100 were selected and synthesized (Table 1). Next, the binding capacities of these peptides to HLA-A*24:02 molecules were tested in MHC stabilization assays using T2-A24 cells. All 10 peptides increased the HLA-A24 expression on the cells, indicating that these peptides bound and stabilized the HLA complex on the cell surface (Table 1). Thus, all 10 peptides were included in the subsequent experiments.

Table 1
Ad11 hexon protein derived peptides predicted to bind to HLA-A24, and the results of the MHC stabilization assays.

Amino acid sequence	Start position	Score[a]	% MFI increase[b]
KYTPSNVTL	482	480	141
DYLSAANML	641	360	77
SYQLLLDSL	366	360	57
LYSNVALYL	469	280	186
VYSGSIPYL	696	200	583
LYANSAHAL	889	200	37
NYNIGYQGF	769	180	142
TYFNLGNKF	37	158	82
NYIGFRDNF	322	150	89
GYKDRMYSF	782	120	27

Ad11, adenovirus serotype 11; MFI, mean fluorescence intensity.
[a] Estimated half-time of dissociation from HLA-A24 (min).
[b] Percent MFI increase of HLA-A*24:02 molecules on T2-A24 cells.

3.2. Induction and expansion of peptide-specific CD8$^+$ T-cells from healthy donor PBMCs

To investigate the immunogenic potential of the 10 candidate peptides, HLA-A*24:02+ PBMCs from five healthy donors, of whom three were seropositive and two were seronegative for Ad11, were stimulated in vitro with each of these peptides. After 3-4 weekly stimulations, peptide-specific CD8$^+$ T-cell frequencies were determined by IFN-γ secretion assays. Of the 10 peptides tested, INF-γ-producing CD8$^+$ T-cells specific for the respective peptides were induced in the 3 peptides, TYFNLGNKF (TYF), VYS-

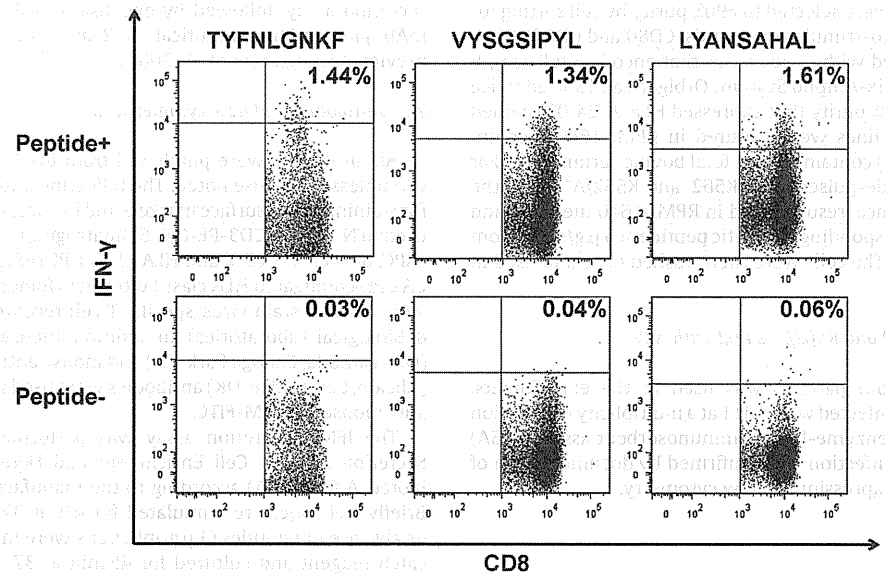

Fig. 1. Induction of peptide-specific CD8$^+$ T-cells from PBMCs of HLA-A*24:02+ healthy donors. PBMCs from five HLA-A*24:02+ healthy donors were stimulated with each of the 10 epitope candidate peptides, and frequencies of IFN-γ-producing CD8$^+$ cells were determined by IFN-γ secretion assays. The numbers in the upper right quadrants are the percentage of IFN-γ-producing cells among CD8$^+$ cells in the presence (upper row) or absence (lower row) of the peptide. Among the 10 peptides tested, 3 induced IFN-γ-producing CD8$^+$ cells.

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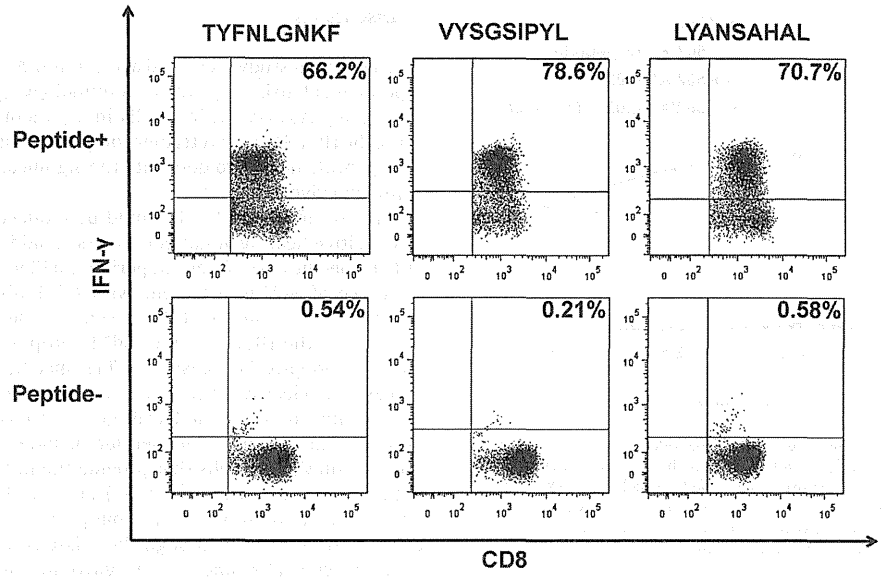

Fig. 2. Enrichment and expansion of peptide-specific CD8+ T-cells. Peptide-specific CD8+ T-cells induced by stimulating PBMCs from HLA-A*24:02+ healthy donors with epitope candidate peptides were isolated using IFN-γ secretion assay and then expanded in the presence of OKT3 mAb, IL-2, and feeder cells. Thereafter, the frequencies of IFN-γ-producing cells among CD8+ cells upon stimulation with autologous LCLs loaded with (upper row) or without (lower row) peptides were determined by intracellular IFN-γ staining assay.

GSIPYL (VYS), and LYANSAHAL (LYA) (Fig. 1). TYF-specific CD8+ T-cells were induced in all three seropositive donors, but not in the two seronegative donors. VYS- and LYA-specific CD8+ T-cells were induced in one of the two seronegative donors. Next, these INF-γ-producing CD8+ T-cells were sorted using an IFN-γ secretion assay, then expanded for subsequent analysis. After sorting and expansion, the frequencies of peptide-specific CD8+ cells among CD8+ cells determined by intracellular cytokine staining assay were above 60% for all three peptides (Fig. 2).

3.3. TYF peptide is naturally processed in Ad11-infected cells and presented in the context of HLA-A*24:02

To determine whether the epitope candidate peptides are naturally processed in Ad11-infected cells and presented by HLA-A*24:02 molecule, the expanded peptide-specific CD8+ T-cells were tested for IFN-γ production against HLA-A*24:02+ cells infected with Ad11. TYF-specific CD8+ T-cells produced significant quantities of IFN-γ against Ad11-infected or TYF peptide-loaded K562/A*24:02, but not against unmanipulated K562/A*24:02 and those loaded with an irrelevant peptide, QYDPVAALF, which is a CMV-pp65 peptide presented by HLA-A*24:02 (Fig. 3A) (Kuzushima et al., 2001). On the other hand, VYS- and LYA-specific CD8+ T-cells did not respond to Ad11-infected K562/A*24:02, indicating that VYS and LYA peptides were not naturally processed and presented on Ad11-infected cells (Fig. 3B, C). Furthermore, TYF-specific CD8+ T-cells effectively lysed TYF peptide-loaded K562/A*24:02 but not TYF peptide-loaded untransfected K562 (Fig. 4). These results indicate that TYF peptide is processed naturally in Ad11-infected cells, presented in the context of HLA-A*24:02, and is an epitope recognized by CD8+ CTLs.

3.4. TYF-specific CTLs do not recognize an HLA-A*24:02 restricted epitope of AdV serotype 5

As the newly identified epitope TYFNLGNKF was similar to the previously identified HLA-A*24:02 restricted epitope of AdV serotype 5 TYFSLNNKF (Leen et al., 2004b), we examined whether

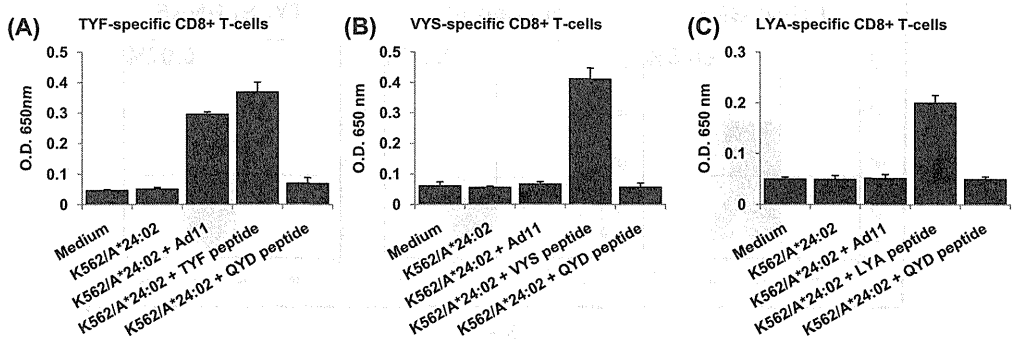

Fig. 3. TYF-specific, but not VYS- and LYA-specific, CD8+ T-cells produce IFN-γ against K562/A*24:02 infected with Ad11. Expanded TYF-specific CD8+ T-cells (A), VYS-specific CD8+ T-cells (B), and LYA-specific CD8+ T-cells (C) were incubated with K562/A*24:02, those loaded with the respective peptides, those infected with Ad11, or those loaded with an irrelevant peptide for 24 h. IFN-γ production in the supernatant was measured by an ELISA. Data are representative of two independent experiments and are the mean ± s.d. of triplicate experiments.

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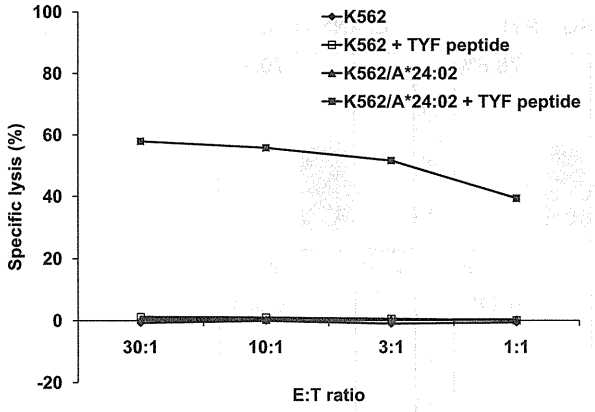

Fig. 4. TYF-specific CD8+ T-cells recognize TYF peptide in the context of HLA-A*24:02 and effectively lyse K562/A*24:02 loaded with TYF peptide. Cytotoxicities of TYF-specific CD8+ T-cells against K562, K562 loaded with TYF peptide, K562/A*24:02, and K562/A*24:02 loaded with TYF peptide were evaluated in ^{51}Cr release assays. Data are representative of two independent experiments and are the mean of triplicate experiments at various E:T ratios.

TYFNLGNKF-specific CD8+ T-cells recognize the TYFSLNNKF peptide. TYFNLGNKF-specific CD8+ T-cells did not produce IFN-γ against autologous LCLs loaded with TYFSLNNKF peptide (Fig. 5), suggesting that TYFNLGNKF-specific CD8+ T-cells are not reactive against AdV serotype 5-infected cells.

3.5. Significance of the TYF epitope in vivo

A PE-labeled HLA-A*24:02 tetramer complexed with the peptide TYFNLGNKF (A*24:02/TYF tetramer) was produced to investigate the significance of the TYF epitope in vivo. The A*24:02/TYF tetramer bound to the TYF-specific CD8+ T-cells but not to the irrelevant T-cells, confirming the specificity of the tetramer constructed (Fig. 6A). We analyzed the frequency of the TYF-specific CD8+ T-cells using A*24:02/TYF tetramers in a HLA-A*24:02+ patient with Ad11 infection. A 64-year-old female with refractory follicular lymphoma developed Ad11-associated HC at 9 days after receiving multiagent chemotherapy. The patient received supportive care, including hydration and pain management, but did not receive any antiviral drugs. In this case, emergence of TYF-specific CD8+ T-cells in peripheral blood coincided with the clearance of Ad11, suggesting that the TYF epitope indeed functions as a target for Ad11-specific CTLs in vivo (Fig. 6B).

4. Discussion

The current study identified a novel HLA-A*24:02 restricted epitope of Ad11 using a reverse immunology approach. Moreover, monitoring Ad11-specific T-cells in a patient with Ad11 disease using the HLA-A*24:02 tetramer complexed with the identified epitope peptide helped to delineate the significance of the identified epitope in vivo.

TYF-specific CD8+ T-cells could be induced in all three Ad11-seropositive healthy volunteer donors. In addition, the emergence of TYF-specific CD8+ T-cells in peripheral blood coincided with the clearance of AdV in a patient with Ad11 disease. These results suggest that TYF-specific CD8+ T-cells can be generated from the majority of the HLA-A*24:02+ Ad11-seropositive healthy donors and that adoptively transferred TYF-specific CD8+ T-cells could successfully clear Ad11 in vivo. Possible drawbacks of adoptively transferring virus-specific T-cells generated by stimulating PBMCs with a single immunogenic peptide include the fact that they do not contain CD4+ T-cells that provide the necessary help to CD8+ CTLs (Moss and Rickinson, 2005) and the fact that the reconstitution of immunity to one epitope may not be sufficient to control AdV disease. Although these issues need to be addressed in future clinical studies, the in vitro and in vivo results of the present study suggests that generating TYF-specific CD8+ CTLs from HLA-A*24:02+ Ad11-seropositive donors is a practical and effective approach to treat Ad11 infections among HLA-A*24:02+ patients.

The newly identified HLA-A*24:02 restricted epitope of Ad11 (subgroup B) was located between amino acid positions 37 and 45 of the hexon protein, which was same as those of AdV serotype 5 (subgroup C) (Leen et al., 2004b). The identified epitope of Ad11, TYFNLGNKF, differed from that of AdV serotype 5, TYFSLNNKF, only in two amino acids, as expected by the fact that the location of these epitopes was within the conserved region of the hexon protein (Ebner et al., 2005). However, TYFNLGNKF-specific CD8+ T-cells, which were reactive against Ad11-infected cells, did not produce IFN-γ against HLA-A*24:02+ LCLs loaded with TYFSLNNKF peptide derived from AdV serotype 5. Similarly, TYFSLNNKF-specific CD8+ T-cells were not reactive against Ad11-infected cells (Leen et al., 2004b). Although previous studies reported that AdV-specific T-cells cross-react with AdV serotypes from different AdV subgroups (Leen et al., 2004b; Tang et al., 2006), these data indicate that AdV-specific T-cells are not necessarily cross-reactive. Thus, determining the subgroup of AdV responsible for infection in individual patients may be necessary before adoptively transferring AdV-specific T-cells.

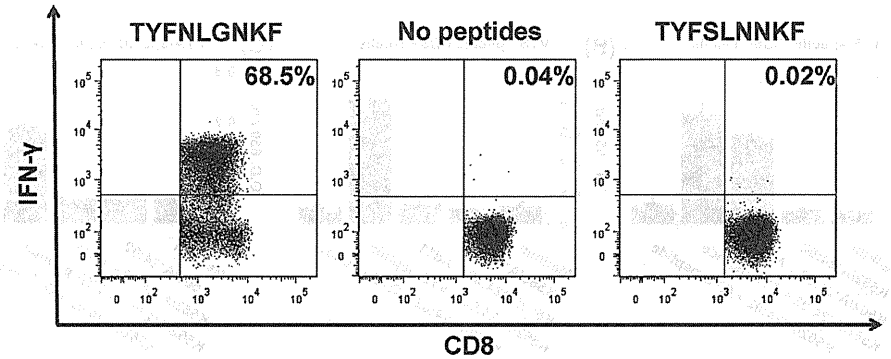

Fig. 5. TYF-specific CD8+ T-cells do not recognize an HLA-A*24:02 restricted epitope of AdV serotype 5. TYF-specific CD8+ T-cells were incubated with autologous LCLs loaded with TYFNLGNKF (a newly identified epitope of AdV serotype 11), those loaded with TYFSLNNKF (a previously identified epitope of AdV serotype 5), or peptide unloaded autologous LCLs. The numbers in the upper right quadrants are the percentage of IFN-γ-producing cells among CD8+ cells upon stimulation. Data are representative of two independent experiments.

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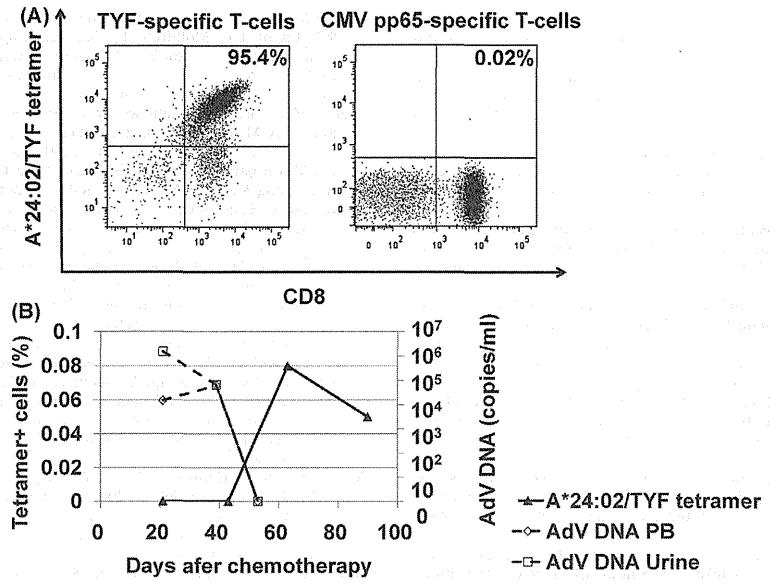

Fig. 6. Frequencies of TYF-specific CD8$^+$ T-cells in a patient with Ad11 disease as determined by staining with HLA-A*24:02/TYF tetramer. (A) TYF-specific CD8$^+$ T-cells and HLA-A*24:02 restricted CD8$^+$ T-cells specific for CMV pp65 peptide QYDPVAALF were stained with PE-labeled HLA-A*24:02/TYF tetramer. Data are representative of two independent experiments. (B) Increase in the percentage of A*24:02/TYF tetramer-positive cells among CD8$^+$ cells coincided with the decrease in adenoviral load in urine and peripheral blood in a patient with Ad11 disease.

As preparation of virus-specific T-cells requires several weeks (Leen et al., 2009), early identification of patients at the risk of developing disseminated AdV disease is crucial. Although previous studies suggested that monitoring AdV-specific cellular immunity using Enzyme-linked immunosorbent spot assays or intracellular cytokine assays by flow cytometry might identify these patients (Guerin-El Khourouj et al., 2011; Myers et al., 2007), these methods are too laborious and complex for routine clinical use. In this regard, the MHC tetramer assay is attractive, because it can rapidly quantify virus-specific CD8$^+$ T-cells using very simple procedures. Previous studies that monitored CMV-specific CD8$^+$ T-cells for prediction of recurrent or persistent CMV infection described the utility of the tetramer assays (Gondo et al., 2004; Gratama et al., 2010). Thus, it is worth exploring whether monitoring Ad11-specific CD8$^+$ T-cells with the A*24:02/TYF tetramer can identify patients at high risk for severe Ad11 disease. In addition, the A*24:02/TYF tetramer can be used to monitor the kinetics of adoptively transferred Ad11-specific T-cells, which is essential for the evaluation of the efficacy of the transferred cells. Taken together, these data suggest that the A*24:02/TYF tetramer is a very useful tool with multiple important applications.

CD8$^+$ T-cells specific for VYS and LYA peptides were induced from one healthy donor. However, these T-cells were not reactive against HLA-A*24:02+ cells infected with Ad11, indicating that VYS and LYA peptides were not naturally processed and presented by HLA-A*2402+ cells infected with Ad11. These peptides are located in the conserved region of the hexon protein (Ebner et al., 2005), and AdV other than serotype 11 have the same or similar amino acid sequence as these peptides in their hexon protein. In addition, the donor from whom VYS- and LYA-specific CD8$^+$ T-cells were induced was seronegative for Ad11. Thus, induced VYS- and LYA-specific CD8$^+$ T-cells might be T-cells specific for other serotypes of AdV.

In the current study, as in the previous studies (Kuzushima et al., 2001), the binding affinity of peptides to MHC molecules determined by a computer algorithm (BIMAS) and that determined by a MHC stabilization assay did not correlate very well. One of the caveats of MHC stabilization assays is that the results would be affected by culture conditions. In this regard, cell free assays capable of directly measuring the binding affinity of peptides to MHC molecules may be beneficial (Liu et al., 2011). In addition, although BIMAS was used to identify potential HLA-A24-binding peptides in the current study, several other epitope prediction algorithms such as one offered at the IEDB website (http://www.iedb.org/) are available. Application of these tools may allow more efficient identification of T-cell epitopes.

In conclusion, we identified a novel HLA-A*24:02 restricted epitope of Ad11, TYFNLGNKF, that could be used to generate Ad11-specific CD8$^+$ T-cells for adoptive immunotherapy. A*24:02/TYF tetramers can be used to monitor Ad11-specific CD8$^+$ T-cell responses in immunocompromised patients at risk for developing Ad11 disease and following adoptive transfer of Ad11-specific CD8$^+$ T-cells.

Conflict of interest

Shingo Toji is a current employee of Medical & Biological Laboratories Co., Ltd. Susumu Suzuki is an advisory role of Medical & Biological Laboratories Co., Ltd.

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